pCRISPomyces-1 and pCRISPomyces-2 Plasmid Assembly Protocol

1) Select a 20 nt protospacer of interest. The 3' protospacer adjacent sequence (PAM) must be NGG, where N is any nucleotide. Preference is given to:
    a. sequences with purines occupying the last four (3') bases of the protospacer.
    b. sequences on the non-coding strand.
    c. sequences in which the last 12 nt of protospacer + 3 nt PAM (15 nt total) are unique in the genome (check by BLAST with all four possible NGG sequences).

2) **For pCRISPomyces-1 only (proceed to step 3 for pCRISPomyces-2)**: Design two 32 nt oligonucleotides with the following sequences:
    a. Spacer-for: 5'-ccaaaac N_{20} gtttt-3'
    b. Spacer-rev: 5'-ctctaaaac N'$_{20}$ gtt-3'

    For example, if the spacer sequence is CTCACGGACGGAGACCAGGA, then the two primers are:
    a. Spacer-for: 5'-ccaaaacCTCACGGACGGAGACCAGGAgtttt-3'
    b. Spacer-rev: 5'-ctctaaaacTCCTGGTCTCCGTCCGTGAGgtt -3'

    Such that the annealed product will be:

    5′-ccaaaacCTCACGGACGGAGACCAGGAgtttt -3′
    3′- ttgGAGTGCCTGCCTCTGGTCCTcaaaatctc-5′

    For plasmids with multiple spacers, design a synthetic construct with the following configuration:

    5'-BbsI-ccaaaac-spacer1-direct repeat-spacer2-gttttagag-BbsI-3'

    Note that the sticky ends for the *Bbs*I sites (5' to 3') are CCAA and AGAG (underlined). Proceed to step 4 (for single spacer) or step 5 (for multiple spacers).

3) **For pCRISPomyces-2 only**: Design two 24 nt oligonucleotides (4 nt 5' sticky end + 20 nt spacer sequence) with the sticky ends ACGC on the forward primer and AAAC on the reverse primer.

    For example, if the spacer sequence is CTCACGGACGGAGACCAGGA, then the two primers are:
    a. Spacer-for: 5'-ACGCCTCACGGACGGAGACCAGGA-3'
    b. Spacer-rev: 5'-AAACTCCTGGTCTCCGTCCGTGAG-3'

    Such that the annealed product will be:

    5′-ACGCCTCACGGACGGAGACCAGGA -3′
    3′- GAGTGCCTGCCTCTGGTCCTCAAA-5′

    For plasmids with two guide RNA cassettes, design a synthetic construct with the following configuration (see example at the end of this document):

    5'-BbsI-acgc-spacer1-sgRNAtracr-T7term-gapdhp(EL)-spacer2-gttt-BbsI-3'

    Note that the sticky ends for the *Bbs*I sites (5' to 3') are ACGC and GTTT (underlined). Proceed to step 4 (for single guide RNA) or step 5 (for multiple guide RNAs).

4) For single spacers, anneal spacer oligos as follows:
    a. Resuspend both oligos to 100µM in water
    b. Mix 5uL FOR + 5uL REV + 90uL 30mM HEPES, pH 7.8
    c. Heat to 95°C for 5min, then ramp to 4°C at 0.1°C/sec

5) Insert annealed spacer (or dual-spacer/guide synthetic construct) to desired plasmid by Golden Gate assembly.

Golden Gate reaction mixture:

Backbone	X µL	100 ng
Insert	0.3 µL	3:1 mol ratio or higher (dilute annealed oligos 10-fold)
T4 Ligase Buffer (NEB)	2 µL	
T4 ligase (NEB)	1 µL	400 U/µL stock is sufficient; add last
BbsI (NEB)	1 µL	Stored at -80 °C
H_2O	Y µL	
	20 µL	

Golden Gate Program:
37°C 10 min
16°C 10 min
Goto step 1, 9 times
50°C, 5 min
65°C, 20 min
4°C, forever

6) Transform 3 µL of each reaction to *E. coli* NEB5alpha by heat shock (manufacturer's protocol).

7) Plate 10% of recovery culture on selective plates with 10uL of 0.5 M IPTG and 40uL of 20mg/mL Bluo-gal (in DMSO).

8) Pick white colonies to selective LB and recover plasmid.

9) Meanwhile, PCR amplify two 1 kb homology arms from genomic DNA of the strain of interest to serve as an editing template. [Note: Make sure that the editing template does not include the full protospacer + PAM sequence!] Two options for primer design:
   a. Design overlaps (20-30 nt) at both ends of both arms and directly perform 3-piece Gibson assembly (1 kb left arm + 1 kb right arm + digested plasmid).
   b. Design overlaps (20-30 nt) at the junction of the two arms and *Xba*I cutsites at the opposite ends of the two arms. Then splice the two arms by overlap-extension PCR, and digest and ligate the 2 kb product into the digested plasmid.

10) Digest the spacer-containing plasmid (assembled in steps 1-7) with *Xba*I. Dephosphorylate with FastAP (Thermo) or similar to prevent re-ligation.

11) Perform the chosen assembly method to insert the 2 kb editing template in your digested, dephosphorylated plasmid.

12) Transform assembly product to NEB5alpha competent cells (or similar).

13) The next day, pick colonies to LB+apramycin.

14) Recover plasmids and confirm by digestion or sequencing.

15) Transform confirmed plasmid to an appropriate conjugation donor strain, and conjugate following standard protocol.

Example synthetic construct for two guide RNAs:

(BbsI-spacer1-gRNAtail-T7term-gapdhp(EL)-spacer2-BbsI)

5’-

GAGACATCTTTGAAGACAAacgcGTTCTCCGCACTCCCATGAGgttttagagctagaaatagcaagttaaaataaggctagtccgtt
atcaacttgaaaaagtggcaccgagtcggtgctttttagcataacccctttggggcctctaaacgggtcttgaggggttttttggctgctccttcggtcgg
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cgcgcaccgggtgcggagcccctcggcgagcggtgtgaaacttctgtgaatggcctgttcggttgctttttttatacggctgccagataaggcttgcagc
atctgggcggctaccgctatgatcggggcgttcctgcaattcttagtgcgagtatctgaaaggggatacgcGCAGACCATGATCAGGGGGAg
tttAAGTCTTCTTTCACGTGGC

-3’